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Calcium signals regulated by NAADP and two-pore channels - their role in development, differentiation and cancer

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ABSTRACT Ca^{2+} signals regulate a wide range of physiological processes. Intracellular Ca^{2+} stores can be mobilized in response to extracellular stimuli via a range of signal transduction mechanisms, often involving recruitment of diffusible second messenger molecules. The Ca^{2+} mobilizing messengers $InsP_3$ and cADPR release Ca^{2+} from the endoplasmic reticulum via $InsP_3$ and ryanodine receptors, respectively, while a third messenger, NAADP, releases Ca^{2+} from acidic endosomes and lysosomes. Bidirectional communication between the ER and acidic organelles has functional relevance for endolysosomal function as well as for the generation of Ca^{2+} signals. The two-pore channels (TPCs) are currently strong candidates for being key components of NAADP-regulated Ca^{2+} channels. Ca^{2+} signals have been shown to play important roles in embryonic development and cell differentiation; however, much remains to be established about the exact signalling mechanisms involved. Investigation of the role of NAADP and TPCs in development and differentiation is still at an early stage, but recent studies have suggested that they play important roles at key developmental stages *in vivo* and are important mediators of differentiation of neurons, skeletal muscle cells and osteoclasts *in vitro*. NAADP signals and TPCs have also been implicated in autophagy, an important process in differentiation. Moreover, potential links between TPC2 and cancer have been recently identified. Further studies will be required to identify the precise mechanisms of action of TPCs and their link with NAADP signalling, and to relate these to their roles in differentiation and other key developmental processes in the cell and organism.

KEY WORDS: *development, differentiation, cancer, calcium, endolysosomal, NAADP, two-pore channel*

Introduction

The generation of new cells by cell division during embryo development is an essential attribute of multicellular life. An equally crucial aspect of development is the commitment of non-specialized embryonic stem cells and their subsequent differentiation into all the cell types of the organism. While less dramatic, growth and differentiation continue in adulthood, as part of the process of tissue repair and during normal cell turnover. Another central factor in embryogenesis is apoptosis, the process of programmed cell death, which shapes the morphology of the embryo during development and regulates cell numbers in adulthood (Lockshin and Zakeri 2007). Autophagy, an intracellular mechanism involving degradation of unused or dysfunctional cellular components, is also central to the maintenance and survival of the organism (Mizushima and Levine 2010, Wu *et al.*, 2013). Abnormalities in growth and differentiation can lead to cancer (Moustakas and Heldin 2007). As such, a better understanding of the mechanisms that control these processes

Abbreviations used in this paper: ARCs, ADP-ribosyl cyclases; β-NAD^+, β-nicotinamide adenine dinucleotide; Ca^{2+}, calcium; $[Ca^{2+}]i$, cytoplasmic free Ca^{2+}; $[Ca^{2+}]i$, luminal Ca^{2+}; CaM, calmodulin; CaMKII, Ca^{2+}/CaM-dependent protein kinase II; cAMP, cyclic AMP; cGMP, cyclic GMP; CICR, Ca^{2+}-induced Ca^{2+}-release; CREB, cAMP response element binding protein; DKO, double knockout; ER, endoplasmic reticulum; ESCs, embryonic stem cells; GPN, glycyl-L-phenylalanine-beta-naphthylamide; Hs, *Homo sapiens*; IP_3, inositol 1,4,5 trisphosphate; IP_3Rs, inositol 1,4,5 trisphosphate receptors; MAPKs, mitogen-activated protein kinases; MEFs, mouse embryonic fibroblasts; MOs, morpholinos; mTOR, mammalian target of rapamycin; NAADP, nicotinic acid adenine dinucleotide phosphate; NCKX, $Na^+/Ca^{2+}/K^+$ exchanger; ncRNAs, non-coding RNAs; NCX, Na^+/Ca^{2+} exchanger; NF-AT, nuclear factor of activated T-cells; NF-κB, nuclear factor-κB; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PKC, protein kinase C; PMCA, Ca^{2+} ATPase; PTP, permeability transition pore; VOCCs, voltage-operated Ca^{2+} channels; ryanodine receptors (RyRs); SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SOCE, store-operated Ca^{2+} entry; STIM1, stromal interaction molecule 1; Sp, *Strongylocentrotus purpuratus*; SR, sarcoplasmic reticulum; MCU, mitochondrial Ca^{2+} uniporter; TPCs, two-pore channels; VEGF, vascular endothelial growth factor; WT, wild type.

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can improve our understanding of how developmental disorders arise, and lead to new methods of diagnosing and treating cancer.

During human embryo development, a single fertilized egg gives rise to an adult containing approximately 100 trillion cells encompassing around 200 different cell types that with a few exceptions (e.g. red blood cells and B- and T-lymphocytes) each contain the same genomic DNA sequence. The many and varied differences in shape, size and functional characteristics of different cell types are due to highly controlled modifications of gene expression. Differences in gene expression in different cell types are due to their possession of different transcription factors, differential methylation of the genome, and to histone modification states (de Pretis and Pelizzola 2014). Increasingly, there is also recognition that non-coding RNAs (ncRNAs) can regulate gene expression (Morris and Mattick 2014). Such epigenetic changes can play both activatory and inhibitory roles in this process (de Pretis and Pelizzola 2014).

Epigenetic control of gene expression is itself tightly linked to the signalling pathways of the organism. On the one hand extracellular signalling molecules, such as hormones, growth factors, cytokines and neurotransmitters, convey messages between different parts of the organism through the blood and extracellular fluid. On the other, intracellular signalling pathways can involve both ionic and chemical changes in the cell. Typically, a second messenger such as cyclic AMP (cAMP) causes changes in the cell by activating a protein effector, in this case protein kinase A, that acts to phosphorylate other target proteins and thereby change their activity (Blumenthal, 2012). Other small molecule second messengers include cyclic GMP (cGMP), inositol 1,4,5 trisphosphate (IP_3), and nicotinic acid adenine dinucleotide phosphate (NAADP); second messengers can also be gases such as nitric oxide, as well as ions, such as calcium ions (Ca^{2+}). It is particularly with the role that Ca^{2+} signals play in the control of differentiation and development that this review will be concerned, and more specifically with the role played by the second messenger NAADP, which recent studies have shown to be a key regulator of a particular type of Ca^{2+} signals emanating from the endolysosomal compartment, via the action of a new class of ion channels called the two-pore channels (TPCs) (Zhu *et al.,* 2010, Patel *et al.,* 2011).

Ca^{2+} signals - modulators of physiological processes

Changes in intracellular Ca^{2+} regulate a diverse range of cellular processes including fertilization, proliferation, differentiation, contraction, secretion, exocytosis, metabolism, gene transcription and apoptosis (Berridge *et al.,* 2003). Cells express a number of effector proteins that bind Ca^{2+} specifically, reversibly and with affinities that allow them to respond to Ca^{2+} at physiological signalling concentrations; these effectors modulate a wide variety of Ca^{2+}-sensitive cellular processes (Carafoli *et al.,* 2001, Clapham 2007). Various conserved Ca^{2+}-binding motifs on proteins give them

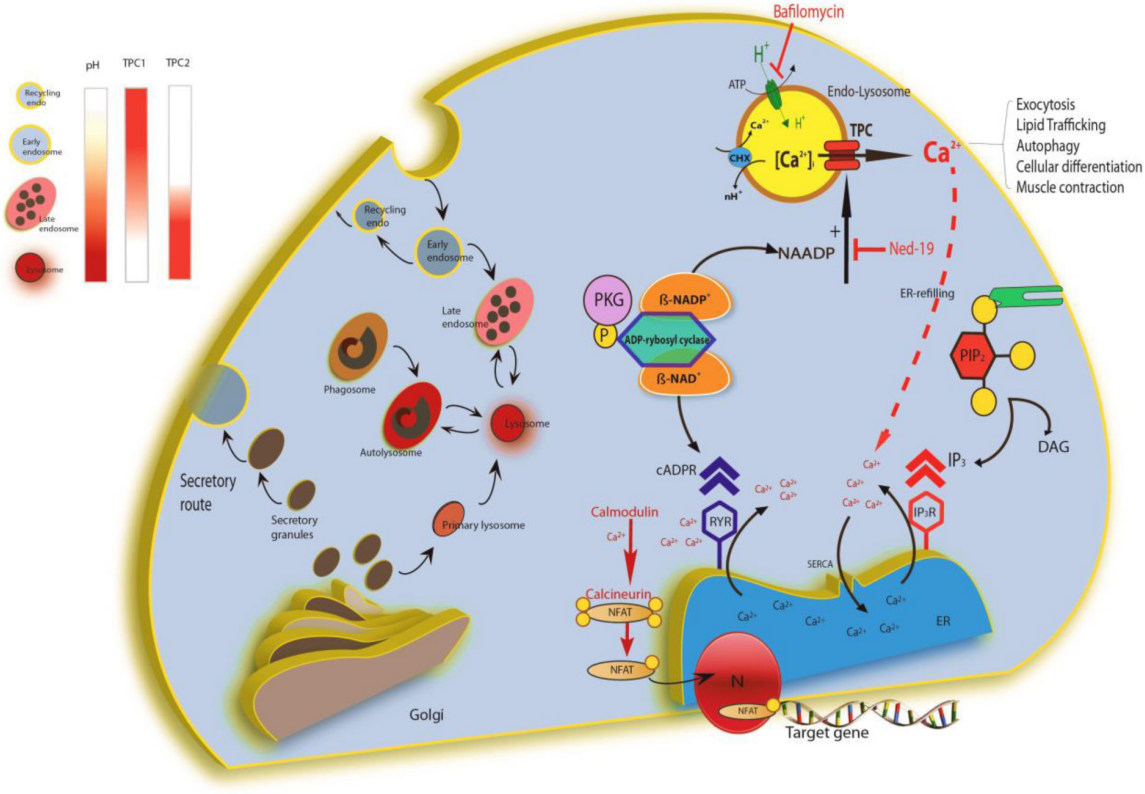

Fig. 1. Schematic representation of a cell depicting Ca^{2+} release from the endoplasmic reticulum (ER) via inositol trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) in the presence of inositol 1,4,5-trisphosphate (IP_3) and cyclic ADP-ribose (cADPR) respectively. *IP_3 is the by-product of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis by phospholipase C (PLC), whereas cADPR is produced from nicotinamide adenine dinucleotide (NAD^+) by ADP-ribosyl cyclase. In the endolysosomal system, NAADP elicits Ca^{2+} release from the acidic stores, lysosomes and endosomes, via the two-pore channels (TPCs). This signalling pathway plays important roles in exocytosis, lipid trafficking, autophagy, cellular differentiation, and muscle contraction. Local Ca^{2+} release by NAADP can trigger global Ca^{2+}-induced Ca^{2+}-release (CICR) responses from the ER or increase ER Ca^{2+} uptake by sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). Changes in intracellular Ca^{2+} ion concentration are also responsible for controlling gene expression, for instance by activation of the transcriptional factor, nuclear factor of activated T-cells (NFAT), by Ca^{2+}-induced protein phosphorylation of calcineurin. The left side of the diagram shows the relative density of TPC1 and TPC2 isoforms in endosomes and lysosomes relative to the level of the acidity in these organelles. Gradients represent relative density of TPC1 or TPC2 or the acidity of the organelles.*

their Ca^{2+} sensitivity. EF-hands are one such motif, being found in a wide range of proteins with a variety of different functions, including regulators of transcription, contraction and apoptosis (Gifford *et al.,* 2007). As well as the direct sensitivity conveyed by an effector protein containing an EF-hand, sensitivity to Ca^{2+} can be achieved indirectly via association with the EF-hand-containing protein calmodulin (CaM), which functionally regulates hundreds of proteins (Carafoli *et al.,* 2001, Clapham 2007). The C2 domain is another Ca^{2+}-binding motif found in many hundreds of proteins including protein kinase C (PKC) and phospholipases; it mediates Ca^{2+}-induced translocation to the plasma membrane (Carafoli *et al.,* 2001, Clapham 2007).

An important feature of Ca^{2+} signals is their range of spatio-temporal properties. Thus, while a single Ca^{2+} spike can trigger Ca^{2+}-sensitive responses such as cardiac contraction and some exocytotic events, more sustained Ca^{2+} signals are required to trigger other processes, such as transcription and fertilization. Such sustained Ca^{2+} signals typically take the form of oscillations, with repeated spikes of increased intracellular Ca^{2+} concentration ($[Ca^{2+}]$i); this minimises the cytotoxicity caused by sustained elevated cytoplasmic free Ca^{2+} and also allows transmission of information at the frequency as well as the amplitude of the signal (Berridge *et al.,* 2003). Such frequency-encoded information is important for the control of gene expression by Ca^{2+}, with different transcription factors being activated by different Ca^{2+} spike frequencies. Thus in Jurkat T-cells, low frequency Ca^{2+} oscillations can activate nuclear factor-κB (NF-κB); however nuclear factor of activated T-cells (NF-AT) and Oct/OAP require higher frequency stimulation for activation (Dolmetsch *et al.,* 1997).

Ca^{2+}/CaM-dependent protein kinase II (CaMKII), which modulates a wide range of cellular processes, has been shown to decode Ca^{2+} oscillation frequency information (De Koninck and Schulman 1998, Coultrap and Bayer 2012). Low frequency Ca^{2+} oscillations result in transient CaMKII kinase activity, while increasing the frequency results in increased levels of sustained activity, which persist after cessation of the Ca^{2+} signal information (De Koninck and Schulman 1998, Coultrap and Bayer 2012). In addition to its effects on transcription factors, CaMKII has recently been shown to exert effects on gene expression through phosphorylation of histones (Kreusser and Backs 2014).

While past studies have focused on the Ca^{2+}-dependent modulation of protein effectors, it is now becoming clear that Ca^{2+} signals can also modulate expression and activity of ncRNAs such as microRNAs (Choi *et al.,* 2014, Gstir *et al.,* 2014, Harada *et al.,* 2014), increasingly recognised as important players in the control of gene expression during embryonic development, by regulating processes such as cell proliferation, differentiation and apoptosis (Moreno-Moya *et al.,* 2014).

Generation of Ca^{2+} signals inside the cell

While a simple ion like Ca^{2+} cannot be modified by common mechanisms of regulation of function such as phosphorylation, changes in Ca^{2+} signals are achieved by altering the balance between Ca^{2+} entry into and extrusion from the site of action, generally the cell cytoplasm, thus leading to a change in the intracellular concentration of Ca^{2+} (Berridge 2001). While $[Ca^{2+}]$i is maintained at around 100nM in resting cells, this can be increased to around 1μM on initiation of Ca^{2+} signalling (Berridge 2001). Sustained elevation of $[Ca^{2+}]$i precipitates phosphate, which is highly detrimental in a system that uses phosphorylation to modulate protein activity (Carafoli *et al.,* 2001, Clapham 2007); it can also activate proteases, leading to cell death (Carafoli *et al.,* 2001). Hence, mechanisms have evolved for removing Ca^{2+} from the cytosol, either by pumping it out of the cell, or sequestering it into intracellular stores. Ca^{2+} binding proteins such as parvalbumin, calsequestrin, calreticulin and calbindin also chelate Ca^{2+}, thus limiting the duration and spatial range of its free diffusion in the cytosol (Berridge 2001, Clapham 2007).

Ca^{2+} is extruded from the cytosol into the extracellular medium by Ca^{2+} ATPase (PMCA), Na^{+}/Ca^{2+} exchanger (NCX) and $Na^{+}/Ca^{2+}/K^{+}$ exchanger (NCKX), all located in the plasma membrane (Berridge 2001). Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), on the other hand, pumps Ca^{2+} into the endoplasmic reticulum (ER), which is present in most mammalian cell types, and in muscle cells into its equivalent, the sarcoplasmic reticulum (SR) (Clapham 2007). The nuclear envelope, an extension of the ER, also sequesters Ca^{2+} via SERCA (Carafoli *et al.,* 2001). Mitochondria also act as buffers and stores of Ca^{2+}, via the action of a number of specific proteins. Thus, Ca^{2+} uptake is mediated by the inner mitochondrial membrane-spanning mitochondrial Ca^{2+} uniporter (MCU) (Hoppe 2010, Pendin *et al.,* 2014), while the mitochondrial Na^{+}/Ca^{2+} exchanger extrudes Ca^{2+} (Nita *et al.,* 2015).

In excitable cells, Ca^{2+} release into the cytosol can occur through plasma membrane voltage-operated Ca^{2+} channels (VOCCs), which undergo conformational changes upon membrane depolarisation, causing them to open and allow Ca^{2+} influx from the extracellular environment. Emptying of intracellular stores can also trigger Ca^{2+} entry across the plasma membrane, in both excitable and non-excitable cells, via store-operated Ca^{2+} entry (SOCE), which produces a Ca^{2+} release-activated Ca^{2+} current (ICRAC) (Lewis 2007, Wu *et al.,* 2007). ER emptying is sensed by the ER membrane protein stromal interaction molecule 1 (STIM1), which responds by coming into close contact with the plasma membrane channel Orai1, activating it and thus causing Ca^{2+} influx (Feske 2007, Lewis 2007, Wu *et al.,* 2007).

Intracellular Ca^{2+} stores can also be mobilised in response to extracellular stimuli via a range of signal transduction mechanisms, often involving diffusible second messenger molecules (Fig. 1). Ca^{2+} release from the endoplasmic reticulum (ER) occurs primarily via $InsP_3$ receptors (IP_3Rs) and ryanodine receptors (RyRs) (Berridge 2001). These can be activated by the second messengers IP_3 and cyclic ADP-ribose (cADPR), respectively, by extracellular stimuli, or by plasma membrane depolarisation in the case of RyR (Fig. 1). Release of Ca^{2+} from the nuclear envelope can also be triggered by IP_3 and cADPR (Carafoli *et al.,* 2001). The lipid sphingosine-1-phosphate may also trigger ER Ca^{2+} release, although its status as a Ca^{2+} mobilizing messenger remains controversial (Young and Nahorski 2002). In addition, the endolysosomal system has recently become recognised as a major Ca^{2+} store (Morgan *et al.,* 2011), a topic that we shall now discuss in detail.

Acidic endolysosomal Ca^{2+} stores and NAADP

The term 'endolysosomal organelles' describes acidic intracellular organelles, including early, late and recycling endosomes and lysosomes; secretory vesicles are also acidic, lysosomal-like organelles (Morgan *et al.,* 2011, Shen *et al.,* 2011). Early endo-

somes are formed by plasma membrane endocytotic events; their lipid constituents may return to the plasma membrane via recycling endosomes, or proceed through the endolysosomal system, forming late endosomes and lysosomes (Fig. 1). During this progression, luminal pH decreases, while $[Ca^{2+}]$l increases, from around 30μM in early endosomes (Gerasimenko *et al.,* 1998; Morgan *et al.,* 2011) to 500-600μM in lysosomes (Christensen *et al.,* 2002, Lloyd-Evans *et al.,* 2008, Morgan *et al.,* 2011). Lysosomes sequester Ca^{2+} in a manner dependent on the low luminal pH of the organelles (Christensen *et al.,* 2002), and disruption of lysosomal Ca^{2+} store mobilisation causes defects in lipid trafficking (Lloyd-Evans *et al.,* 2008). The exact mechanism of endolysosomal Ca^{2+} uptake remains to be fully defined, but is likely to involve SERCA/PMCA-like ATPases (Morgan *et al.,* 2011) and exchangers exploiting the proton gradient of acidic stores (Christensen *et al.,* 2002). This is generated by the vacuolar H^+ATPase; in addition, although simple Ca^{2+}/H^+ exchangers are absent from the mammalian genome, Ca^{2+}/Na^+ exchangers might operate, with Na^+ gradients generated by coupled Na^+/H^+ exchange (Patel and Docampo 2010, Morgan *et al.,* 2011). Some Ca^{2+} also appears to be taken up into the endolysosomal system from the extracellular environment via endocytosis (Christensen *et al.,* 2002, Morgan *et al.,* 2011).

The evidence that Ca^{2+} signals generated from acidic, endolysosomal stores play important physiological roles has come primarily from studies of the second messenger NAADP, which have been carried out in both sea urchin eggs and egg homogenates, and in mammalian cells and tissues (Galione *et al.,* 2011). Such studies have shown that NAADP is a highly potent Ca^{2+}-releasing agent, being active at low nanomolar concentrations (Chini *et al.,* 1995, Lee and Aarhus 1995). Unlike cADPR- and $InsP_3$-mediated Ca^{2+} release, NAADP-induced Ca^{2+} release is insensitive to changes in cytosolic Ca^{2+} concentration or Ca^{2+} chelation, indicating that the target receptor for NAADP is not subject to Ca^{2+}-induced Ca^{2+}-release (CICR) (Chini and Dousa 1996). In urchin egg homogenates, NAADP-evoked Ca^{2+} release is not affected by blockers of cADPR- or IP_3- induced Ca^{2+} release, 8-NH_2-cADPR or heparin (Lee and Aarhus 1995), ER Ca^{2+} depletion by thapsigargin (Genazzani *et al.,* 1996), or prior Ca^{2+} release by cADPR or IP_3 (Chini *et al.,* 1995), but is desensitised by prior NAADP-induced Ca^{2+} release (Lee and Aarhus 1995). In addition, prior exposure to non-Ca^{2+}-releasing concentrations of NAADP has been shown to desensitise urchin egg homogenates to subsequent release by activating concentrations (Genazzani *et al.,* 1996). A small molecule-specific inhibitor of NAADP-mediated Ca^{2+} release, Ned-19, has also been developed (Naylor *et al.,* 2009); studies of its binding properties indicate that the NAADP receptor possesses two NAADP binding sites, a high-affinity inhibitory site and a low affinity activatory site (Rosen *et al.,* 2009), thus providing an explanation for the inhibitory effect of subthreshold NAADP.

The possibility that NAADP might target a Ca^{2+} store other than the ER was first indicated by the finding that in urchin eggs stratified by centrifugation NAADP-sensitive Ca^{2+} stores migrate to the opposite pole to cADPR- and IP_3-sensitive stores (Lee and Aarhus 2000). Subsequently, NAADP-sensitive Ca^{2+} stores, but not IP_3- or cADPR-sensitive stores, were shown to be sensitive to disruption of the lysosomal proton gradient via blockers of the vacuolar H^+ATPase such as bafilomycin A1, and to osmotic lysis of lysosomal stores by glycyl-L-phenylalanine-beta-naphthylamide (GPN), indicating that NAADP releases Ca^{2+} from lysosome-related acidic organelles (Churchill *et al.,* 2002). NAADP-induced Ca^{2+} release was also found to be accompanied by an increase in lysosome luminal pH (Morgan and Galione 2007). Lysosome-related stores were subsequently confirmed as the target of NAADP in mammalian cells (Kinnear *et al.,* 2004, Yamasaki *et al.,* 2004). NAADP can trigger Ca^{2+} release from endosomes as well as lysosomes (Menteyne *et al.,* 2006).

Despite this separation of stores, in some cell types NAADP-evoked Ca^{2+} signalling has been shown to be abrogated by depletion of ER Ca^{2+} stores with thapsigargin or blocking of IP_3R and/or RyR-mediated signalling (Cancela *et al.,* 1999, Santella *et al.,* 2000, Churchill and Galione 2001, Boittin *et al.,* 2002). These findings have led to the 'NAADP trigger hypothesis', whereby Ca^{2+} released by the action of NAADP can recruit IP_3R and RyR-mediated signalling pathways via CICR (Churchill and Galione 2001). Differences in the extent of ER store involvement in different cell types is likely to be a consequence of different patterns of localisation of the stores and receptors, with recruitment occurring when NAADP-mediated Ca^{2+} release occurs in close proximity to ER channels (Kinnear *et al.,* 2004, Kinnear *et al.,* 2008, Morgan *et al.,* 2011). NAADP can also potentiate ER Ca^{2+} release by providing Ca^{2+} that is sequestered, thus increasing ER loading and sensitising RyRs and IP_3Rs via their sensitivity to $[Ca^{2+}]$I (Morgan *et al.,* 2011).

Conversely, recent findings indicate that Ca^{2+} released from the ER can activate the NAADP pathway, both by stimulating Ca^{2+}-dependent NAADP synthesis and by activating NAADP-regulated Ca^{2+} channels (Morgan *et al.,* 2013). This effect may be dependent on local microdomains of high $[Ca^{2+}]$i at junctions between the ER and acidic organelles. Such contact between the ER and acidic organelles has been identified both in sea urchin eggs (Morgan *et al.,* 2013) and in human fibroblasts (Kilpatrick *et al.,* 2013). Bidirectional communication between these different organelles may have functional relevance for endolysosomal function as well as for the generation of Ca^{2+} signals, with one model predicting that different arrangements of Ca^{2+} signalling proteins on the membranes of the two organelles might generate functionally heterogeneous Ca^{2+} microdomains (Penny *et al.,* 2014).

Two-pore channels as NAADP-regulated Ca^{2+} channels

Based on the characteristics of NAADP-induced Ca^{2+} signalling described above, candidate NAADP-regulated Ca^{2+} channels would be expected to possess the following properties: ability to form a functional Ca^{2+} channel; localisation to the endolysosomal compartment; capacity to bind NAADP with all the characteristics displayed by the native NAADP receptor; sensitivity to pharmacological agents that inhibit NAADP-evoked Ca^{2+} signalling, e.g. Ned-19, or to high concentrations of L-type Ca^{2+} blockers; and to be functionally inhibited by lysosomal disruption, e.g. with bafilomycin A1 or GPN (Galione *et al.,* 2009). In addition, an NAADP-regulated Ca^{2+} channel should be insensitive to IP_3R and RyR agonists and antagonists, and to ER store depletion by SERCA inhibitors; however, due to the 'trigger' effect of NAADP-evoked Ca^{2+} signalling seen in some cell types, an effect of these agents on the total response to NAADP might still be observed (Galione *et al.,* 2009). Based on these criteria, TPCs (gene name *Tpcn*), members of the voltage-gated cation channel superfamily (Ishibashi *et al.,* 2000), are currently the strongest candidates for being key components of NAADP-regulated Ca^{2+} channels (Brailoiu *et al.,* 2009, Calcraft

et al., 2009, Zong *et al.,* 2009) (Fig. 2).

Humans and mice have two functional TPC isoforms, TPC1 and TPC2; a third isoform, TPC3, present in many vertebrates including rabbits and chickens (Ogunbayo *et al.,* 2015), is the product of a pseudogene that does not encode a functional protein in humans, Old World monkeys, apes, mice or rats (Calcraft *et al.,* 2009, Cai and Patel, 2010). Cai and Patel (2010) have characterised the evolutionary process that led to the degeneration of the TPC3 gene and loss of its protein's function in the primate lineage. TPC1 and TPC2 are widely expressed in mammalian tissues at the level of mRNA (Ishibashi *et al.,* 2000, Brailoiu *et al.,* 2009, Calcraft *et al.,* 2009, Zong *et al.,* 2009). Immunoblot and immunohistochemistry analysis have also shown that TPC1 protein is expressed at different levels in a variety of tissues in rodents (Ishibashi *et al.,* 2000, Ruas *et al.,* 2014). Subcellular localisation of exogenously expressed and endogenous TPCs has been studied. TPC2 is localised predominantly to late endosomes and lysosomes, while TPC1 exhibits a more general endolysosomal distribution in recycling endosomes, early and late endosomes, and lysosomes (Galione *et al.,* 2011) (Fig. 1). Chicken TPC3 is localised to recycling endosomes and as yet unidentified organelles (Zhu *et al.,* 2010). In human cytotoxic T-cells, endogenous TPCs have been shown in immunofluorescence studies to localise to the cytolytic granules, secretory vesicles that are exocytosed during the cell-killing response; moreover, TPCs play important functional roles in this response (Davis *et al.,* 2012). In line with their localization to the endolysosomal compartment, TPCs have recently been shown to be involved in receptor trafficking and endolysosomal function (Grimm *et al.,* 2014; Ruas *et al.,* 2014). Deficiencies in endolysosomal trafficking *in vivo* have metabolic consequences. Thus TPC2 knockout mice are highly susceptible to hepatic cholesterol overload and liver damage consistent with non-alcoholic fatty liver hepatitis, likely due to abnormal hepatic cholesterol handling (Grimm *et al.,* 2014). Also in line with a role for TPCs in endolysosomal function, infection of mouse cells by Ebola virus, which is taken up by macropinocytosis, followed by trafficking through endosomal vesicles, was inhibited by loss of TPC2 protein, suggesting that TPCs may be effective targets for anti-Ebola virus therapy (Sakurai *et al.*, 2015).

As well as exhibiting the localisation to acidic stores expected of an endogenous NAADP receptor, TPCs also show the same specific pharmacology and sensitivity to NAADP as that demonstrated for an endogenous receptor. Thus, sensitivity to nanomolar NAADP is conveyed by expression of *Homo sapiens* (Hs) TPC1 in SKBR3 cells; this response is sensitive to bafilomycin A1 and ryanodine, suggesting coupling of lysosomal Ca^{2+} release to RyRs via CICR (Brailoiu *et al.,* 2009). In HEK293 cells expressing recombinant HsTPC2, NAADP at nanomolar and low micromolar concentrations induces Ca^{2+} release at levels significantly above the endogenous response; this response is blocked by bafilomycin A1 and reduced by inhibition of RyRs, $InsP_3Rs$, or SERCA, again suggesting an extent of functional coupling of stores (Calcraft *et al.,* 2009). Millimolar NAADP concentrations do not elicit such a response, and anti-TPC2 small hairpin RNAs (shRNAs) inhibit endogenous responses to activated NAADP (Davis *et al.,* 2012). Similarly, endogenous NAADP-induced Ca^{2+} release in SKBR3 cells was inhibited by anti-TPC1 shRNAs (Brailoiu *et al.,* 2009). In line with TPCs being related to voltage-gated Ca^{2+} (Cav) and Na^+ channels (Nav), molecular docking studies of TPC1 suggested that the pore region of this protein has the potential to bind Cav and Nav antagonists; such antagonists also inhibited NAADP-induced Ca^{2+} signals (Rahman *et al.,* 2014).

Importantly for the status of TPCs as a credible candidate for the endogenous NAADP target channel, Ca^{2+} release in response to NAADP has been found to be abrogated in pancreatic β-cells from TPC2 KO mice (Calcraft *et al.,* 2009). Investigation of HsTPC2 reconstituted in a lipid bilayer has indicated that the channel is activated by nanomolar and low micromolar NAADP, but inhibited by micromolar concentrations (Pitt *et al.*, 2010). Binding of NAADP to TPC2 appears to occur at two sites, a high-affinity activating site and a low-affinity inhibitory site, as expected of an endogenous NAADP receptor (Rosen *et al.,* 2009). Similar reconstitution of HsTPC1 in a lipid bilayer has allowed a biophysical comparison of the two isoforms. Using such an approach, Rybalchenko *et al.,* (2012) showed that TPC1 functions as a NAADP-activated and voltage-, pH-, and Ca^{2+}-regulated channel. Activation of TPC1 by NAADP was shown to be dynamically regulated by the membrane potential. Another study of TPC1 reconstituted in a lipid bilayer confirmed that TPC1 activity is stimulated by NAADP, and showed that TPC1 and TPC2 differ in their ion selectivity and modulation by Ca^{2+} and pH, with TPC1 being much more permeable to protons (Pitt *et al.,* 2014). This has led to the suggestion that NAADP

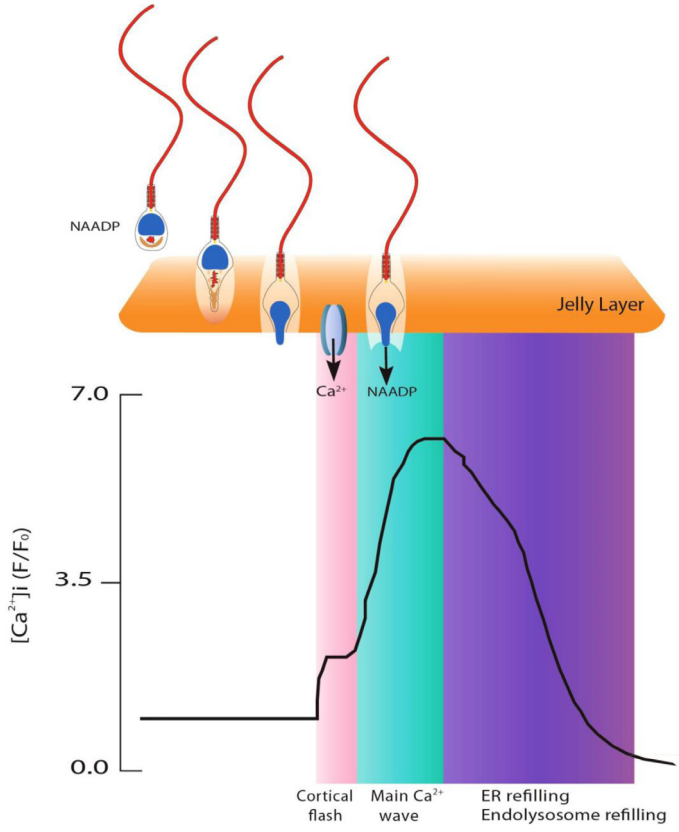

Fig. 2. Fertilization induces Ca^{2+} release in sea urchin eggs. *The fusion of a sea urchin sperm with the egg generates a transient, NAADP-induced cortical 'flash' of Ca^{2+} influx, marking the beginning of the egg activation process. The main Ca^{2+} wave at fertilization is due to Ca^{2+} release from the egg's internal stores in response to three Ca^{2+} mobilizing messengers - IP3, cADPR and NAADP. Ca^{2+} is taken back into the ER via SERCA, and by the endolysosomal vesicles via the calcium-proton exchanger (CHX).*

triggers H^+ release from lysosomes and endolysomes via TPC1, but that the Ca^{2+}-releasing ability of TPC1 depends on the ionic composition of the acidic stores and might be influenced by other regulators that affect TPC1 ion permeation (Pitt *et al.,* 2014).

As much of the early investigation of NAADP-mediated Ca^{2+} signalling was conducted in sea urchin egg systems, a study thoroughly characterising *Strongylocentrotus purpuratus* (Sp) TPCs provides important evidence for TPCs as an endogenous NAADP receptor (Ruas *et al.,* 2010). In this study, NAADP binding was shown to be increased in sea urchin egg fractions containing high levels of endogenous SpTPCs. NAADP bound to immunoprecipitated SpTPCs with high specificity and nanomolar affinity. Binding was irreversible in high $[K^+]$ conditions, an established feature of NAADP binding to endogenous receptors in sea urchins but not in mammals (Dickinson and Patel 2003). Finally, exogenous expression of SpTPC1 and SpTPC2 in HEK293 cells resulted in Ca^{2+} release in response to NAADP, in a manner sensitive to bafilomycin A1 and to desensitising concentrations of NAADP and Ned-19 (Ruas *et al.,* 2010). Another study that expressed SpTPC1 and SpTPC2 in SKBR3 cells also showed that this led to enhanced NAADP-induced Ca^{2+} signalling, as did expression of SpTPC3 (Brailoiu *et al.,* 2010). In contrast Ruas *et al.,* (2010) found that expression of SpTPC3 in HEK293 cells not only did not lead to enhanced NAADP-induced Ca^{2+} signalling but it actually inhibited the endogenous NAADP response in this cell type. The reason for these discrepancies relating to SpTPC3's role in NAADP-induced Ca^{2+} release remains unclear. In summary, a compelling body of evidence suggests that TPCs are an endogenous target of NAADP.

Despite these many and varied findings in support of a link between TPCs and NAADP-mediated Ca^{2+} signalling, the claim that TPCs are NAADP-regulated Ca^{2+} channels has been challenged by a study by Wang *et al.,*2012), which showed that pancreatic β-cells from mice described as TPC1/TPC2 double knockouts still retained the ability to release Ca^{2+} in response to NAADP. The study also reported that electrophysiological analysis of TPCs in cells in which the lysosomes had been swollen with vacuolin showed that TPC1 and TPC2 were primarily Na^+ channels regulated by the lysosomal lipid phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2). A further study by a related group confirmed the ability of TPCs to conduct Na^+ (Cang *et al.,* 2013).

However, two recent studies have confirmed the link between TPCs and NAADP-mediated Ca^{2+} signalling. The first of these studies has shown that while recombinant TPC2 can conduct Na^+ in response to PI(3,5)P2, it can also be activated by NAADP, leading to Ca^{2+} release from endolysosomal stores; the same study also identified Mg^{2+} and the mitogen-activated protein kinases (MAPKs) c-Jun N-terminal kinase (JNK) and P38 as novel regulators of TPC2 (Jha *et al.,* 2014). The second study, by Ruas *et al.,*2015) reaffirmed the status of TPCs as NAADP-regulated Ca^{2+} channels with the first analysis of the biophysical properties of *endogenous* mammalian TPCs and investigation of the effects of eliminating TPC expression on NAADP-evoked Ca^{2+} signalling, in the only *definitively demonstrated* TPC knockout mouse cells studied to date. Importantly, this study of mouse embryonic fibroblasts (MEFs) derived from TPC1/2 double knockout (DKO) mice showed that NAADP-evoked Ca^{2+} signalling was lacking in the absence of TPCs. Moreover, NAADP-evoked Ca^{2+} signalling was restored in the DKO MEFs by expression of either mouse TPC1 or TPC2 recombinant protein, but not by expression of a TPC2 pore mutant or transient receptor potential cation channel, mucolipin subfamily, member 1 (TRPML1), another endolysosomal ion channel previously proposed as a candidate NAADP-regulated Ca^{2+} channel. In addition, truncated forms of mouse TPC1 and TPC2 proteins predicted to be expressed in the putative TPC1/2 DKO cells studied by Wang *et al.,* (2012) were found to be functional, casting doubt on whether those cells had a true loss of TPC function. Importantly, the effects of endogenous TPC loss were not only assessed by single-cell Ca^{2+} imaging after addition of cell-permeant NAADP-AM to wild type (WT) and DKO MEFs, but also by electrophysiological analysis carried out by patch-clamping of single endolysosomes. This latter analysis confirmed that endogenous TPCs are directly able to conduct Ca^{2+} into and out of the endolysosomal compartment in response to NAADP (Ruas *et al.,* 2015).

Despite multiple lines of evidence implicating TPCs as NAADP-regulated Ca^{2+} channels, it still remains unclear how TPCs interact with NAADP. On the one hand immuno-affinity purification of endogenous sea urchin TPCs has shown that such purified TPCs are associated with the ability to bind NAADP with the same high affinity as the native urchin NAADP receptor (Ruas *et al.,* 2010). However, other recent studies have suggested that NAADP may require an intermediary protein to regulate TPC-mediated Ca^{2+} release. Thus, studies employing [^{32}P-5-azido]NAADP as a photoaffinity probe have identified small molecular weight proteins that appear to be high-affinity NAADP-binding protein(s) that are unrelated to TPCs, in both mouse pancreas (Lin-Moshier *et al.,* 2012) and sea urchin egg homogenates (Walseth *et al.,* 2012). The sea urchin egg NAADP-binding protein(s) have been shown to be associated with immunoprecipitated TPCs, suggesting a physiological interaction between these proteins and TPCs. It has also been shown that the ability of urchin TPCs to bind NAADP is mediated by such an accessory protein (Walseth *et al.,* 2012). Importantly, in the TPC DKO mice studied by Ruas *et al.,* (2015) that had a demonstrated loss of expression of both TPC1 and TPC2, high-affinity NAADP binding was still present, in line with such binding being mediated by a protein other than a TPC. An urgent task now is to identify such an NAADP-binding protein at the molecular level, to characterise the nature of its interaction with TPCs, and to show how the interaction relates to the function of TPCs *in vivo*.

Role of NAADP and TPCs during fertilization and embryogenesis

The importance of Ca^{2+} signalling in embryonic development is well established (Berridge 2001). Ca^{2+} signals play important roles throughout embryogenesis, having been implicated in such key developmental stages as fertilization (Parrington *et al.,* 2007, Wakai *et al.,* 2011, Nomikos *et al.,* 2012, Nader *et al.,* 2013), control of cytokinesis (Webb *et al.,* 1997), coordination of complex cell movements during gastrulation (Wallingford *et al.,* 2001), formation of left-right asymmetry (Yoshiba and Hamada 2014), skeletal muscle formation (Cheung *et al.,* 2011), and development of the nervous system (Leclerc *et al.,* 2012). To date, most studies of NAADP and TPC function during embryogenesis have focused on their role as mediators of Ca^{2+} release during activation of the egg at fertilization. This reflects the fact that NAADP, like cADPR, was first identified as a Ca^{2+}-release agent in sea urchin eggs, with the sea urchin egg homogenate having proved to be a very important system

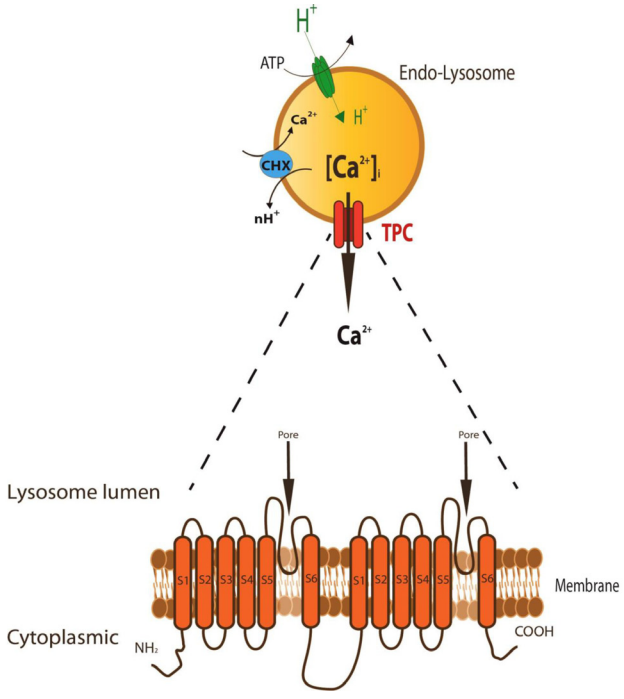

Fig. 3. Schematic drawing of endolysosomal membrane shows the localization of the transmembrane topology of the two-pore channels (TPCs). *There are two putative pore-forming repeats. Each of these repeats contains six transmembrane segments and an intervening pore-loop.*

for the study of the mechanism of action of these Ca^{2+}-mobilising messengers (Lee 2003). NAADP has been shown to be involved in mediating the cortical flash that occurs at fertilization in sea urchins and starfish and contributing to the Ca^{2+} wave that activates the egg, leading to a block to polyspermy and the activation of embryogenesis (Santella *et al.,* 2012) (Fig. 3). The first direct link between endogenous TPCs and NAADP-induced Ca^{2+} signalling was shown by the finding, mentioned above, that immuno-purified sea urchin TPCs are associated with high-affinity NAADP binding (Ruas *et al.,* 2010). This study also showed that urchin TPCs are localised to the cortex of the egg, the site of NAADP-induced Ca^{2+} release during fertilization. Like mammalian TPC1 and TPC2, expression of urchin TPC1 and TPC2 in HEK cells led to greatly enhanced NAADP-induced Ca^{2+} release; however, surprisingly urchin TPC3 *inhibited* endogenous NAADP-induced Ca^{2+} release in HEK cells, suggesting that it can act in a dominant negative manner (Ruas *et al.,* 2010). In contrast, however, another study in which a slightly different variant of urchin TPC3 was expressed in a different cell line did not observe such inhibitory properties for this TPC3, the expression of which was instead shown to enhance NAADP-induced Ca^{2+} release, similar to the urchin TPC1 and TPC2 also assessed in this study (Brailoiu *et al.,* 2010). Interestingly, a recent study of rabbit and chicken TPC3 isoforms has found no evidence that either of these vertebrate TPC3 isoforms can act in a dominant negative manner, although expression of chicken TPC3 did not lead to enhanced NAADP-induced Ca^{2+} release, unlike expression of rabbit TPC3, which did (Ogunbayo *et al.,* 2015).

Other recent studies have investigated the role of urchin ADP-ribosyl cyclases (ARCs), the enzymes that have been shown to have the capacity to generate both cADPR and NAADP *in vitro*, and are therefore believed to fulfil the same role *in vivo* although this remains to be fully confirmed. Cloning of an urchin ARC and analysis of its enzymatic activity after expression in *Xenopus* oocytes showed that it could carry out both the base-exchange and cyclization reactions that generate NAADP and cADPR respectively, even though it had a luminal expression in the ER in this expression system (Churamani *et al.,* 2007). In another study endogenous urchin ARCs were shown to be localised within the cortical granules of the egg (i.e., the acidic exocytotic vesicles involved in the cortical reaction that occurs during egg activation), and such ARCs were shown to be optimally active at the same acidic pH (Davis *et al.,* 2008). This intraorganellar localisation is the first to be demonstrated for an enzyme that generates an intracellular messenger active in the cytosolic compartment and poses the question of how ARC substrates might gain entry to, and ARC products leave, the organelle. At least for cADPR, the study showed that the substrate for cADPR synthesis, β-NAD^+, enters the acidic organelle through a β-NAD^+ transporter, while cADPR exits the organelle through a separate cADPR transporter. The physiological importance of ARCs for egg activation was shown by the fact that inhibition of these transporters inhibited the fertilization-induced Ca^{2+} wave (Davis *et al.,* 2008).

To gain insights into the functional roles of endogenous TPCs and ARCs during not just fertilization but also further development of the echinoderm embryo, a recent study investigated the role of ARCs and TPCs during fertilization in the starfish (Ramos *et al.,* 2014). In contrast to sea urchins, in which three ARC isoforms were identified (Churamani *et al.,* 2007; Davis *et al.,* 2008), the starfish was found to only have a single ARC isoform (Ramos *et al.,* 2014). In addition, like the urchin which has three TPC isoforms (Brailoiu *et al.*, 2010; Ruas *et al.,* 2010), the same number of these were identified in starfish (Ramos *et al.,* 2014). Immunolocalisation analysis showed that the TPCs and one ARC isoform were all localised to the cortical region. However, while all three TPCs were present in the cortical granules, as in the urchin, the starfish ARC was not found in cortical granules but in a different, unknown set of vesicles closely apposed to the cortical granules (Ramos *et al.,* 2014). Intriguingly, immunogold electron microscopy indicated that starfish TPCs were localised to internal membranes within the cortical granules, posing questions about how this pattern of localisation allows them to function as Ca^{2+} channels in this organelle. Assuming that other pumps, exchangers and channels are present in the cortical granule inner membranes, one possibility is that separation of membrane-bound compartments within the cortical granules allows coupling of electrochemical gradients (as happens in mitochondria) (Ramos *et al.,* 2014).

To study the functional role of ARCs and TPCs during starfish fertilization and embryogenesis, morpholinos (MOs) have been used to inhibit their expression (Ramos *et al.,* 2014). Knockdown of TPC2 and TPC3 was shown to lead to abnormalities in the formation/elevation of the fertilization envelope. This result is consistent with the suggestion that TPCs play a functional role in the cortical granules, which make a major contribution to the formation of this envelope. Although individual knockdown of TPCs or ARC did not result in changes in the shape, timing or amplitude of Ca^{2+} dynamics at fertilization, simultaneous knockdown of all three TPC isoforms did lead to substantial abnormalities in the fertilization Ca^{2+} wave, indicating a role for TPCs in this process. Demonstrating the importance of ARC and TPC function to embryogenesis in starfish,

knockdown of TPC2, TPC3, or ARC, led to embryonic lethality at the gastrula stage; in contrast, knockdown of TPC1 did not have any effects upon embryonic development (Ramos *et al.,* 2014).

In vertebrates, *Xenopus* ARC CD38, and also ARC activity, were shown to be developmentally regulated during embryogenesis of this species (Churamani *et al.,* 2012). Moreover, this study also demonstrated the functional importance of this protein for embryogenesis, since chemical or molecular inhibition of CD38 abolished ARC activity and disrupted elongation of the anterior-posterior axis and differentiation of skeletal muscle, culminating in embryonic death (Churamani *et al.,* 2012). Evidence for an important role for TPCs during embryonic development in vertebrates comes from a recent study that has investigated the role of TPC2 during development of slow muscle cells (SMCs) in the intact zebrafish embryo (Kelu *et al.,* 2015). Ca^{2+} signals have previously been shown to accompany differentiation of SMCs during *in vivo* zebrafish development (Cheung *et al.,* 2011). The recent study, in a transgenic zebrafish line that expresses the Ca^{2+}-sensitive bioluminescent protein aequorin specifically in skeletal muscle cells, found that knockdown of TPC2 with MOs resulted in a dramatic attenuation of Ca^{2+} signals and a significant disruption of myofibrilogenesis (Kelu *et al.,* 2015). Moreover, treatment of embryos with Ned-19, the specific antagonist of NAADP-induced Ca^{2+} release, or bafilomycin A1 also disrupted both Ca^{2+} signalling and myofibrilogenesis. An MO-resistant recombinant version of zebrafish TPC2 partially rescued the normal pattern of SMC Ca^{2+} signals (Kelu *et al.,* 2015). This suggests that NAADP-induced Ca^{2+} release via TPC2 plays an important role during SMC development in the zebrafish embryo (Fig. 4). It will be important for future studies to investigate the mechanisms whereby such Ca^{2+} signals regulate muscle development in this manner.

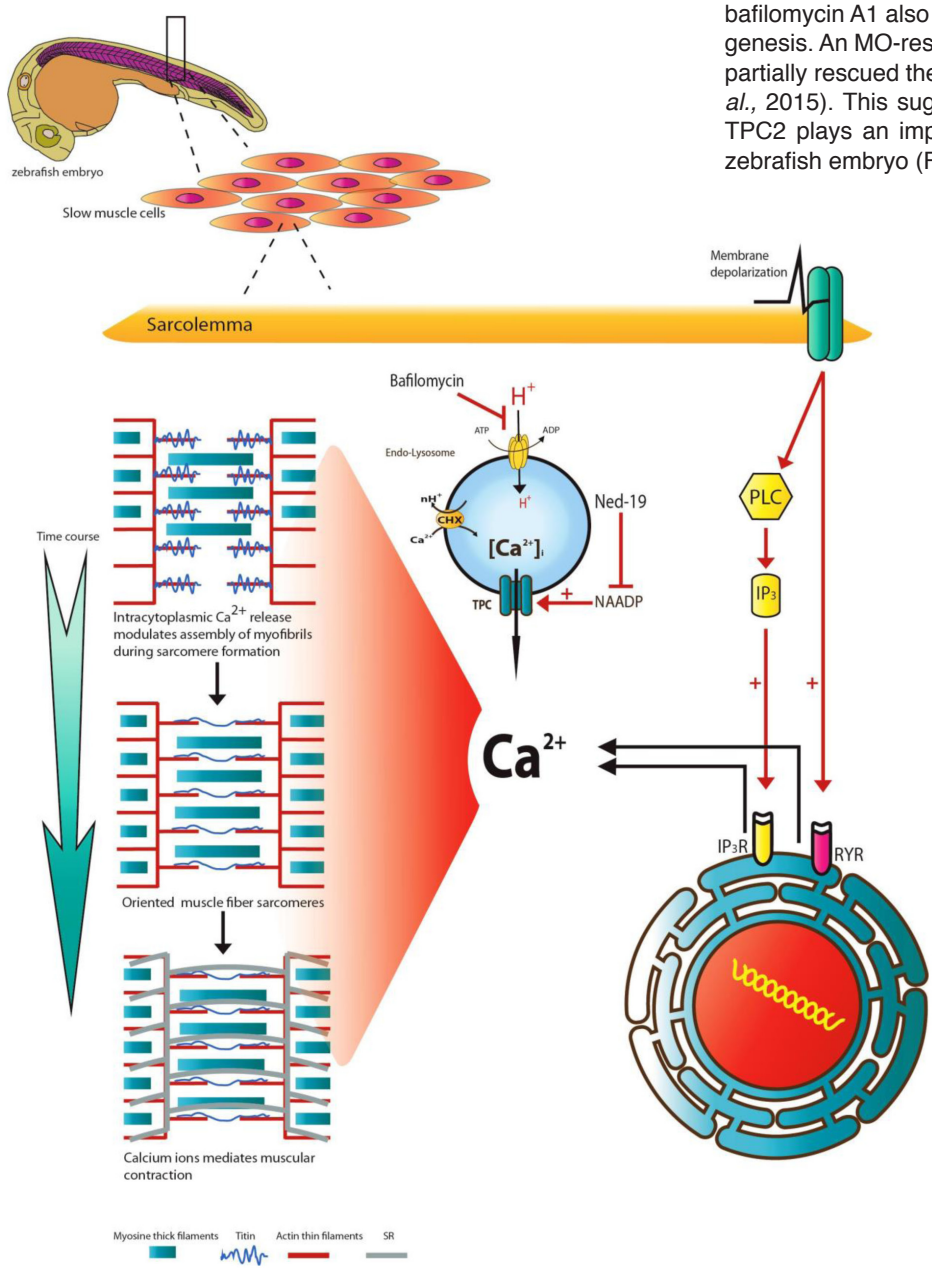

Fig. 4. Schematic diagram of the role of NAADP and TPCs during development of slow muscle cells (SMCs) in zebrafish embryos. *Treatment with bafilomycin A1, Ned-19 or anti-TPC2 morphlinos disrupts Ca^{2+} signalling and myofibrilogenesis.*

Whether TPCs have functional roles during mammalian fertilization and embryonic development remains to be properly investigated. TPC single and double knockout mice are fertile and loss of TPC1 and TPC2 has no effect on embryonic viability or the ability to progress to adulthood (Calcraft *et al.,* 2009, Ruas *et al.,* 2014, Ruas *et al.,* 2015). However given the capacity of mouse embryos to compensate for loss of expression of important Ca^{2+} signalling proteins by 'remodelling' other signalling pathways (Berridge *et al.,* 2003), this does not exclude the possibility of important roles for TPCs during mammalian reproduction and embryogenesis. Indeed, a recent study that investigated the role of NAADP and TPCs in sperm biology found that NAADP could activate the acrosome reaction, whereas this response was blocked by treatment with Ned-19, as well as being absent in TPC1 knockout mice (Arndt *et al.,* 2014). The study also found that NAADP binding sites and TPC1 showed co-localisation in the acrosomal region, providing further support for the idea that TPCs are NAADP-regulated Ca^{2+} channels. Interestingly, two narrow bell-shaped dose-response curves were identified with maxima in either the nanomolar or low micromolar NAADP concentration range, with TPC1 being found to be responsible for activating the low affinity pathway by means of analysis of the TPC1 knockout (Arndt *et al.,* 2014). This raises the question of whether the high affinity pathway involves TPC2, an issue that remains to be explored.

We still lack detailed information about the spatial and temporal expression of TPCs

during mammalian embryonic development. However one study has investigated the expression of TPC1 and TPC2 in embryonic muscle during development and found that both isoforms are expressed in this tissue at embryonic stages E17 and E18 (Aley *et al.,* 2010). Indeed, expression of both isoforms, but particularly of TPC2, was much more pronounced in the embryo than in the adult. Recent studies of TPC knockout mice have shown them to have abnormalities in smooth muscle contraction (Tugba Durlu-Kandilci *et al.,* 2010), receptor trafficking and endolysosomal function (Ruas *et al.,* 2014), storage and utilization of cholesterol by the liver (Grimm *et al.,* 2014), angiogenesis (Favia *et al.,* 2014), skeletal muscle function (Cang *et al.,* 2013, Lin *et al.,* 2014), brown adipose tissue thermogenesis (Lear *et al.,* 2014) and heart contraction in response to β-adrenergic stimulation (Capel *et al.,* 2015). Although these abnormalities may reflect defects in the signalling pathways regulating physiological responses in the affected tissues, it is also possible that they are due to disturbances of TPC expression during development. Since all of these studies are of whole-animal knockouts, it will be important in the future to generate inducible knockouts in which loss of TPC expression can be triggered at different stages of embryogenesis or development to adulthood. In addition, since the phenotype of a particular cell type or tissue in a whole animal knockout might not reflect the effects of a defect in TPC function in a different part of the organism, it will also be important in the generation of such inducible TPC knockouts to include those that are cell-type specific (conditional), so that the effects of loss of TPC expression in a specific cell type or tissue can be compared with loss of expression in the whole organism.

Role of NAADP and TPCs during commitment and differentiation

The processes of commitment and terminal differentiation are central to the formation of specialised cell types during embryogenesis and continue to be important during adulthood in repair and regeneration. A number of studies have highlighted the central role that Ca^{2+} signals play during specialisation of a variety of different cell types. The vast majority of these studies, however, have exclusively used *in vitro* models and have not distinguished between commitment (or, more properly, *in vitro* surrogate specification) and terminal differentiation. Ca^{2+} transients that occur in the first few days of multipotent neural crest cell specialisation have been shown to be required for neuronal differentiation *in vitro* (Carey and Matsumoto 1999). Skeletal muscle fibre differentiation also requires Ca^{2+} signals, with the spatiotemporal pattern being an important factor in fibre type specialisation; thus prolonged elevation of intracellular Ca^{2+}-induces slow-twitch fibre differentiation, while short, high amplitude transients favour fast-twitch fibre formation (Chin *et al.,* 1998). In B lymphocytes, Ca^{2+} signals initiate changes in gene expression and cellular events including apoptosis and differentiation (Baba and Kurosaki 2011). Ca^{2+} signals also appear to regulate many important aspects of keratinocyte differentiation, for instance the formation of desmosomes, adherens junctions and tight junctions, which maintain cell-cell adhesion, although, again, many of the data are derived from *in vitro* models (Bikle *et al.,* 2012). Specialisation requires the expression of cell type-specific genes, and Ca^{2+} signals modulate this through regulation of transcription factors, such as cAMP response element binding protein (CREB) (Shaywitz and Greenberg 1999) and NF-AT (Hogan *et al.,* 2003), and Ca^{2+} signal transducers, such as the protein phosphatase calcineurin and CaM-dependent protein kinases (CaMKs) (Shaywitz and Greenberg 1999, Ikura *et al.,* 2002, Hogan *et al.,* 2003). Thus, CaMK and calcineurin/NF-AT signalling have been shown to drive transcription of myogenin, a key regulator of myogenesis (Xu *et al.,* 2002, Armand *et al.,* 2008), while disruption of intracellular Ca^{2+} signalling (Porter *et al.,* 2002) or global *in vivo* NF-AT deletion (Kegley *et al.,* 2001) inhibits muscle differentiation. The pattern of Ca^{2+} signals has been shown to be important in determining the response elicited in specialising cells – in neuron precursor cells, Ca^{2+} transients of a narrow range of frequencies affect phenotype development through modulation of neurotransmitter expression and channel maturation, while waves of elevated intracellular Ca^{2+} drive axon outgrowth (Gu and Spitzer 1995). In 3T3-L1 preadipocyte cells (Ntambi and Takova 1996) and primary adipose-derived human preadipocytes (Shi *et al.,* 2000), increasing intracellular Ca^{2+} with an ionophore or thapsigargin within the first 24-48 hours of differentiation has been shown to inhibit adipogenesis, as judged by lipid accumulation and expression/activity of mature adipocyte markers. One caveat with such treatments is that they might also interfere with endogenous Ca^{2+} signalling by depleting stores and therefore mask highly specific signalling. The inhibition was still observed in the presence of a Ca^{2+} chelator (Ntambi and Takova 1996), suggesting that it was store depletion rather than increased intracellular Ca^{2+} that was having the effect. When intracellular Ca^{2+} levels were increased by such means during later differentiation, (i.e., between 48-72 hours after induction), the effect on adipogenesis was positive (Shi *et al.,* 2000). This might indicate a role for Ca^{2+} in promoting terminal differentiation; however, it is again difficult to draw conclusions as the pharmacologically-induced Ca^{2+} rises are unlikely to mimic the spatiotemporal patterns of physiological signals that might be occurring during commitment and differentiation *in vivo*.

Recent studies have implicated NAADP- and TPC-mediated Ca^{2+} signalling in the specialisation of several cell types. Liposome-mediated delivery of NAADP to PC12 cells, a model for neuronal differentiation, resulted in Ca^{2+} release and development of neuronal morphology (Brailoiu *et al.,* 2006). Induction of differentiation was also triggered by cADPR-mediated, but not IP_3-mediated, Ca^{2+} release (Brailoiu *et al.,* 2006). Another study suggested that TPC2 plays a dual role during commitment/specification and differentiation of mouse embryonic stem cells (ESCs) into neurons; thus expression of TPC2 was markedly decreased during generation of neural progenitors, but gradually increased again during later stages of neural differentiation (Zhang *et al.,* 2013). In line with such a dual role, knockdown of TPC2 accelerated ESC specification into neural progenitors but inhibited these from differentiating into neurons, while overexpression of TPC2 inhibited ESCs from becoming specified to the early neural lineage (Zhang *et al.,* 2013).

As already mentioned, inhibition of the ARC CD38 during *Xenopus* embryogenesis inhibited muscle differentiation (Churamani *et al.,* 2012). Membrane-permeant NAADP-acetoxymethyl ester (AM) has been shown to promote myogenic differentiation in primary skeletal muscle precursor cells and in the C2C12 myogenic cell line, while inhibition of NAADP-induced Ca^{2+} release with Ned-19, bafilomycin A1 or desensitising NAADP concentrations inhibits differentiation, as assessed by morphological and molecular markers (Aley *et al.,* 2010). While TPC1 mRNA expression was found to be relatively constant throughout the course of myocyte

terminal differentiation in these systems, TPC2 mRNA expression was highest in the first few days of differentiation and substantially reduced thereafter, indicating the possibility of distinct roles for the two isoforms during the process. siRNA-mediated knockdown of TPC1 in C2C12 cells caused moderate impairment of differentiation, while TPC2 knockdown caused substantial inhibition (Aley *et al.,* 2010). Recently, TPC2 has also been implicated in osteoclast differentiation (Notomi *et al.,* 2012). Expression of TPC2 mRNA was upregulated in primary bone marrow stromal cells (measured after 5 days of differentiation) and an osteoclast precursor cell line (measured after 3 days of differentiation) when osteoclastogenesis was induced; moreover, miRNA-mediated TPC2 knockdown disrupted Ca^{2+} signalling and inhibited differentiation. These effects could be mediated by NF-AT, as TPC2 knockdown has been shown to dramatically inhibit nuclear translocation of this transcription factor (Notomi *et al.,* 2012). Another recent study has found that extracellular application of NAADP affects osteoclast formation, demonstrating a potential role for NAADP-induced Ca^{2+} signalling in this process (Cheng *et al.,* 2015). Most recently, extracellularly applied NAADP also stimulated differentiation of human epidermal keratinocytes into terminally differentiated cells, as assessed by an increase in mRNA transcripts associated with keratinocyte differentiation (Park *et al.,* 2015). Such extracellular NAADP treatment also enhanced the migration and proliferation of human epidermal keratinocytes in an *in vitro* wound scratch assay, and wound closure in an *in vivo* mouse model (Park *et al.,* 2015).

NAADP is thought to be generated *in vivo* by the ADP-ribosyl cyclase CD38 (Cosker *et al.,* 2010), which also generates cADPR (Lee 2012). CD38 has been implicated in differentiation of osteoclasts (Sun *et al.,* 2003) and hematopoietic cells (Orciani *et al.,* 2008). CD38 has been shown to be present in the nucleus (Adebanjo *et al.,* 1999) and NAADP has been shown to trigger nuclear Ca^{2+} signals in pancreatic acinar cells (Gerasimenko *et al.,* 2003), while depolarization of *Aplysia* neurons triggers the translocation of CD38 to the nucleus (Bezin *et al.,* 2008). Nuclear Ca^{2+} signals play a role in the differentiation of cardiomyocytes (Janowski *et al.,* 2006), and retinoic acid-induced differentiation of HL-60 myeloblastic cells is associated with the appearance of active CD38 in the nucleus (Yalcintepe *et al.,* 2005). In future studies, therefore, it will be important to characterise mechanistically the links between NAADP- and TPC-mediated Ca^{2+} release and nuclear Ca^{2+} signals, as well as to ascertain the role of the latter in the regulation of commitment and/or differentiation.

Role of NAADP and TPCs in autophagy

Autophagy is an evolutionarily-conserved catabolic degradation process utilised by eukaryotic cells and tissues in response to environmental stresses such as starvation and disease (Sandri 2010, Choi *et al.,* 2013) and it also plays important roles in development and differentiation (Mizushima and Komatsu 2011, Zois *et al.,* 2011, Murrow and Debnath 2013). The fact that autophagy involves degradation by the autolysosome, which is formed by fusion of an autophagasome and a lysosome, suggests that NAADP signalling and TPCs might be involved in this process. In line with this, introduction of NAADP into rat astrocytes not only induces Ca^{2+} signals from acidic organelles in these cells, but also increases levels of the autophagic markers LC3II and beclin-1 (Pereira *et al.,* 2011). The NAADP-induced increases in LC3II levels were reduced in cells expressing a dominant-negative TPC2 construct. NAADP-induced Ca^{2+} signalling mediated by TPC2 may either regulate autophagy by triggering subsequent ER Ca^{2+} release which then activates Ca^{2+}/calmodulin-dependent kinase-β and AMP-activated protein kinase, or by local Ca^{2+} release events that promote fusion of autophagosomes and lysosomes (Pereira *et al.,* 2011).

In another study, overexpression of TPC2 in HeLa or mouse ES cells inhibits autophagosomal-lysosomal fusion, thereby resulting in the accumulation of autophagosomes (Lu *et al.,* 2013). Knockdown of TPC2 or treatment of cells with the NAADP antagonist Ned-19 also markedly decreases the accumulation of autophagosomes (Lu *et al.,* 2013). In two other studies, regulation of autophagic flux by dynein-mediated autophagosomes has been shown to be inhibited by Ned-19 (Xu *et al.,* 2013, 2014). Mice lacking CD38 (the enzyme that can generate cADPR and NAADP) also show defects in autophagosome trafficking and consequently impaired autophagic flux in coronary arterial myocytes (Xiong *et al.,* 2013, Zhang *et al.,* 2014).

Presenilins are required for efficient proteolysis in autophagy, a failure in this mechanism being linked to the pathogenesis of several major neurodegenerative diseases, particularly Alzheimer's disease (Nixon and Yang, 2011). Absence of presenilin expression has been shown to be associated with abnormalities in lysosomal Ca^{2+}, as well as changes in TPC expression and level of dimerization, which might be factors in the disruption of autophagy in presenilin-null cells (Neely Kayala *et al.,* 2012). TPC2 has also been shown to interact physically with Hax-1, a negative regulator of autophagy and apoptosis (Lam *et al.,* 2013). Most recently, a study of skeletal muscle in TPC2 knockout mice has shown that they have abnormal autophagic flux in their skeletal muscle. Thus muscle derived from such mice shows exacerbated autophagy during starvation (Lin *et al.,* 2014). This study also showed that TPC2 is associated with the nutrient sensor, mammalian target of rapamycin (mTOR), in skeletal muscle, suggesting that TPC2 may modulate mTOR reactivation during autophagy and therefore contribute to muscle homeostasis. An association between human TPCs and mTOR was identified by a recent study of the TPC 'interactome' (Lin-Moshier *et al.,* 2014). Another recent study also identified an important role for TPCs in muscle function linked to its association with mTOR (Cang *et al.,* 2013). In line with this, the study reported that mice characterised as TPC1/2 DKO animals did not have gross differences in autophagy, but they did have severely reduced endurance after fasting. One caveat with this study was that the mice studied were the same animals investigated by Wang *et al.,* (2012), whose DKO status has recently been questioned by Ruas *et al.,* (2015). In particular, and as already discussed, the truncated TPC1 and TPC2 forms predicted to be expressed in those mice may in fact be partially or wholly functional (Ruas *et al.,* 2015). Nevertheless, the study suggests that interfering with TPC expression does have significant effects upon muscle function during nutrient deprivation, and thus provides further evidence of a role for TPCs in nutrient sensing.

Role of NAADP and TPCs in cancer

The association between growth, differentiation and cancer is well known. Development of the embryo from a fertilized egg

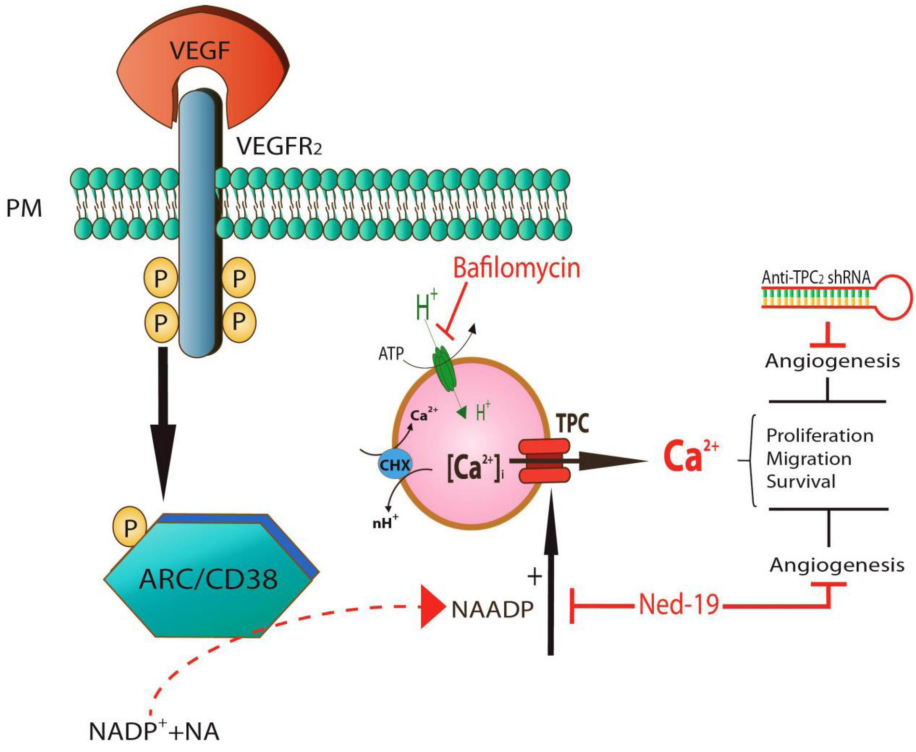

Fig. 5. Schematic representation of the role of NAADP-regulated Ca^{2+} signalling in VEGF-mediated angiogenesis. *Treatment with Ned-19, anti-TPC2 shRNAs, or TPC2 gene knockout abolishes VEGF-induced angiogenic responses.*

to a multicellular organism of many different cell types results from the precise regulation of growth and differentiation, as does repair and regeneration of tissues and organs in the adult. It is not surprising then that abnormalities in the signalling pathways controlling these processes can lead to a cancerous phenotype (Berridge *et al.,* 2003). The reported links between NAADP and TPCs and the control of growth and differentiation therefore raise the question of whether abnormalities in NAADP-regulated Ca^{2+} signalling and TPC action can lead to cancer. Exploration of this issue is still in its infancy; however a number of studies have begun to identify potential links between TPC2 and cancer. Thus, two studies of genetic factors underlying the development of growth and metastasis in oral squamous cell carcinoma in humans have identified over-expression of the TPC2 gene as a potential driver of the amplification of a genomic region, 11q13, observed in different tumours (Huang *et al.,* 2006, Sugahara *et al.,* 2011). What remains to be identified is the mechanism whereby over-expression of a lysosomal ion channel such as TPC2 could lead to changes in growth or metastatic potential of such tumours.

An association between TPC2 and cancer has also emerged from studies investigating the link between genes involved in skin pigmentation and a tendency for malignant melanoma. A number of such studies have identified polymorphisms in the TPC2 gene as potential risk factors for this condition (Pho and Leachman 2010, Kosiniak-Kamysz *et al.,* 2014). Here, there is a clearer mechanistic rationale for TPC2 involvement, since the melanosome that secretes skin and hair pigment is a modified lysosome (Sturm 2009). Indeed, polymorphisms in the TPC2 gene have been shown to be associated with skin and hair colour in humans (Sturm 2009). It is possible then that changes in TPC2 expression or function lead to abnormalities in melanocyte biology that might potentiate tumorigenesis in this cell type.

In addition to these potentially direct links between TPC2 and cancer, a recent study has identified an important role for NAADP-regulated Ca^{2+} signalling and TPC2 during angiogenesis (Favia *et al.,* 2014). This process, whereby new blood vessels form from pre-existing vessels, is a normal and crucial aspect of embryonic development, and plays an important role in wound healing in the adult. However, angiogenesis is also a fundamental step in the transition of tumours from a benign to a malignant state. Vascular endothelial growth factor (VEGF) and its receptors VEGFR1 and VEGFR2 play major roles in controlling angiogenesis, including vascularization of solid tumours. The role of NAADP-regulated Ca^{2+} signalling in VEGF-mediated angiogenesis has now been demonstrated by the finding that treatment with Ned-19 or anti-TPC2 shRNA abolishes VEGF-induced Ca^{2+} release and *in vitro* angiogenic responses in human umbilical vein endothelial cells (HUVECs) (Favia *et al.,* 2014). *In vivo*, moreover, VEGF-induced vessel formation in matrigel plugs was abolished by Ned-19 and failed to occur in TPC2 knockout, but not in TPC1 knockout, mice (Favia *et al.,* 2014). These findings suggest that pharmacological modulation of NAADP-induced Ca^{2+} signals mediated by TPC2 might represent an important future anti-cancer therapeutic strategy (Fig. 5).

Concluding remarks

Recent studies have highlighted the importance of Ca^{2+} signals regulated by NAADP acting on acidic endolysosomal organelles as important mediators of cellular function. Numerous studies over recent years have implicated TPCs, a family of endolysosomal proteins, as NAADP-regulated Ca^{2+} channels. In particular, a recent study has investigated the biophysical properties of endogenous mammalian TPCs, in the only definitively demonstrated TPC knockout mouse cells studied to date, and reaffirmed the status of TPCs as NAADP-regulated Ca^{2+} channels. Ca^{2+} signals have been shown to play important roles during numerous stages of embryogenesis, and in the differentiation of several key cell types. However, much remains to be established about the exact signalling mechanisms involved at different developmental stages. Analysis of the role of NAADP and TPCs in development and differentiation is still in its infancy, but recent studies suggest that they are important in embryonic development in echinoderms and as mediators of differentiation in neurons, skeletal muscle cells and osteoclasts in vertebrates. NAADP-induced Ca^{2+}signals and TPCs have also been implicated in autophagy, a process implicated in differentiation. Finally, recent studies have identified links between TPC2 and cancer, and shown that TPC2 plays a role in angiogenesis, a

key developmental process that becomes subverted during tumour survival and metastasis. Further studies will be required to identify the precise mechanisms of TPC action and their links with NAADP signalling, and to relate these to the roles of TPCs in development, differentiation and other key processes in the cell and organism.

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